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# Nutmeg (*Myristica fragrans*) Of North Minahasa Molecular Entication Based on chloroplast DNA of matK gene

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**Abstract**

Plant Nutmeg (Myristica fragrans) is an endemic plant in Indonesia originating from Banda Maluku Province. Nutmegs have long been known to people as spices. The nutmeg plants have been cultivated in North Sulawesi for a long time. This research has been conducted to obtain the position of species and the reconstruction of phylogenetic tree of nutmeg plant (Myristica fragrans) from North Minahasa based on chloroplast DNA of matK gene. This research uses descriptive method with laboratory experiment. Total DNA was obtained by leaf tissue extraction and purification. The matK gene was amplified using PCR method and visualized with automatic electrophoresis, sequencing was performed at FIRST BASE Singapore. The result of the analysis of the gene sequence of North Min Minerals using Barcode Of Life Database (BOLD) Systems shows that Pala Wori and Pala Patokaan have 99% similarity with Myristica maingayi and Myristica glubosa. While the reconstruction of the phylogeny tree shows the North Minahasa Pala has the closest resemblance to Myristica fatua.

**Key words:** Pala *(Myristica fragrans),* Gen *mat*K, *Barcode Of Life Database* (BOLD) Systems

## 1. INTRODUCTION

Nutmeg (*Myristica fragrans*) is an endemic plant originating from Banda Maluku Province, has long been known as a spice material and as an important commodity in the geopolitical field (Heyne, 1987). Nutmeg is an important spice and is widely used as a flavor enhancer and traditional medicine. Nutmeg plants as spices give a specific aroma and are widely used in the pharmaceutical field, especially a number of lipid contents. Since the beginning until now nutmeg

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has remained one of the promising cultivation plants in the tropics because it has high economic value (Mckee and Harden, 1991).

North Minahasa Regency is one of the nutmeg production centers in North Sulawesi Province. Nutmeg producers are almost scattered throughout the North Minahasa Regency and part of the population depends on the agricultural sector, especially on nutmeg commodities (Anonymous, 2012). Nutmeg is included in the family Myristicaceae reportedly a multipurpose plant, which in addition to being used as a spice and traditional medicine, turned out to have a antiplatelet aggregating, antifungal, hypolipidaemic, anti-ulcerogenic, anxiogenic, and anti-inflammatory activity carminative, (Morita *et al.*, 2003).

Information on plant diversity and types of nutmeg varieties is still limited. The information is important as a conservation of representation of existing diversity as a basis for plant breeding for the development of new high yielding varieties. Nutmeg germplasm conservation is also needed to prevent genetic erosion due to various human actions in the location of nutmeg germplasm growth, one of which is by DNA barcode (Hutammi, 2006).

DNA barcode is identification using a molecular approach that is based on short DNA fragments (Hebert *et al.*, 2003). An organism can be identified and differentiated by barcode DNA starting from the species stage to sub species. DNA barcoding data are also useful for inventorying species diversity and taxonomic identification (Lahaye *et al.*, 2008).

The advantage of this technique is to identify two characterizations of various species that cannot be distinguished morphologically (Tudge, 2000). In addition this technique is also used to identify the organism's status even though the DNA of the organism is not in whole or pure form, even the DNA has been degraded (Hajibabaei, 2006). The use of two plastid genes namely rbcL and matK is recommended by the Consortium for the Barcode of Life (CBOL) as a standard barcode for plants and cytochrome oxidase sub unit 1 (COI) for animals (Hollingsworth *et al.*, 2009). At present, the rbcL and matK genes have been used as DNA DNA for plant species (Hebert *et al.*, 2003). Compared to chromosomal genes (nucleus genes) DNA chloroplasts have an evolutionary rate of 1-10x faster. This study aims to obtain the position of species and the phylogeny tree reconstruction of the Nutmeg (*Myristica fragrans*) plant from North Minahasa based on DNA chloroplast matK gene.

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## 2. MATERIAL AND METHODS

### 2.1 Materials

Nutmeg leaves and fruits are obtained from two nutmeg plantation areas in North of Minahasa namely Wori and Patokaan. Samples were taken in the morning at 08.00 - 09.00. The sample was preserved in a sample box at a temperature of 25$^0$C. Before extracting, the sample was determined to ensure that the nutmeg plant was used (Figure 1).

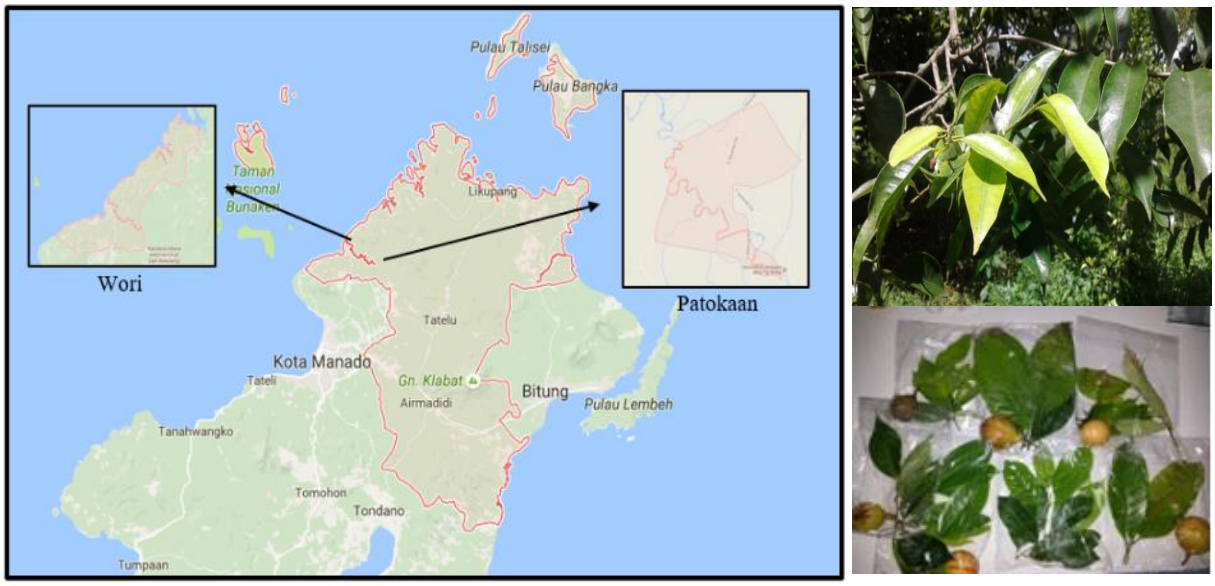


Figure 1. Nutmeg Sampling Location

The tools used in this study were 1.5 ml eppendorf microsphere centrifuge, eppendorf micropipette, vortex, ice board, 2 ml collection tube, GD column, centrifuge, Qi-Rotor Qene Rotor, QIAxcel Advanced Qiagen, UV / VIS Spectrometer Lambda 35 Perkin Elmer. The material in this study uses Genomic DNA Mini KIT (Plant) Geneaid terdiri dari buffer GP1, Buffer GPX1, Rnase A, buffer GP2, buffer GP3, buffer W1, wash buffer, elution buffer (TE), MyTaq HS Red Bioline, DNA templete (DNA Pala), primer *mat*K_f (5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3') and primer *mat*K_rev (5'-ACC CAG TCC ATC TGG AAA TCT TGG TTC-3').

### 2.2 Methods

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This research uses descriptive research method. The research data were obtained through laboratory experimental activities. Stages of research include: total DNA extraction and purification, target gene amplification, amplification visualization, sequencing (Figure 2).

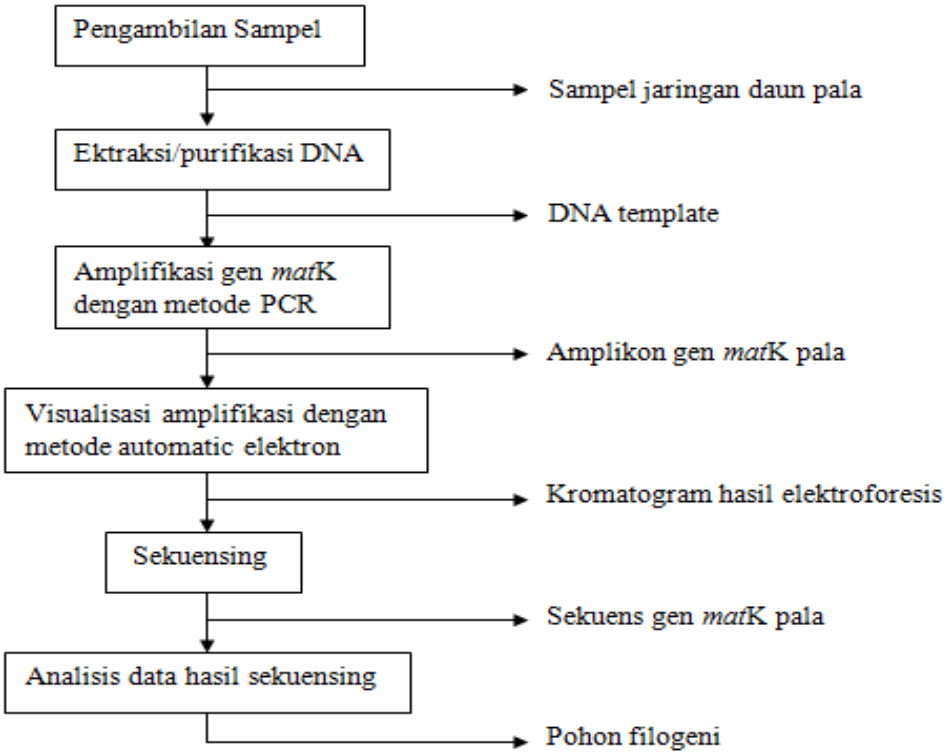


Figure 2. Research Flow Diagram

## C. Research Procedure

### 1. Extraction and Purification of total DNA Nutmeg

***Dissociation.*** Cut tissue samples (nutmeg leaves) 50 mg (max 100 mg) for fresh samples or frozen samples or 10 mg (max 25 mg) for dry samples. Then mash the sample into powder then transfer to a 1.5 ml microsentrifuse tube.

***Lysis.*** Add 400µl GP1 buffer or GPX1 buffer and 5µl Rnase A to the sample in the tube then vortex. After that, incubate at 60ºC for 10 minutes, while incubating the tubes shake every 5 minutes. During this time the pre-heat elution buffer (200 µl per sample) at 60 ˚ C (to be used in step 5). Then add 100 µl GP2 buffer and mix with vortex then incubate using ice (use the ice board in the referigrator) for 3 minutes. Place the column filter in a new 2 ml collection tube then move the mixture in the column filter. Then centrifuge for 1 minute at 13,200rpm then lift the column

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filter. After that, carefully remove the supernatant from the collection tube in the new 1.5 ml microsentrifuse tube.*DNA*

***Binding.*** Add 1.5 volumes of GP3 buffer then vortex quickly for 5 seconds. Then place the GD column in the 2 ml collection tube. Then transfer 700 µl of the mixture (and all remaining precipitation) to the GD column. After that centrifuge at 13,200 rpm for 2 minutes. Then lift the GD column and place it back on the 2 ml collection tube. Add the remaining mixture to the GD column then centrifuge at 13,200 rpm for 2 minutes. Then lift the GD column and place it back on the 2 ml collection tube.

***Washing.*** Add 400 µl of W1 buffer to the GD column then centrifuge at 13,200 rpm for 30 seconds. Lift the GD column and place it back on the 2 ml collection tube. Add 600 µl Wash buffer to GD column. Then centrifuge at 13,200 rpm for 30 seconds. Lift the GD column and put it back on the 2 ml collection tube. Then centrifuge for 3 minutes at 13,200 g to get a dry column matrix.

***Elution.*** Transfer the GD column to the dry column on a new 1.5 centrifuge tube., Measure 100 µl pre-heated elution buffer (TE) in the middle of the matrix column. Then leave it for 3-5 minutes to ensure the elution buffer (TE) is fully absorbed. Then centrifuge at 13,200 rpm for 30 seconds for DNA purification.

**2. Analysis of Purity and DNA Concentration**

The results of total mtDNA extraction were then analyzed for concentration and purity using a nanospectrophotometer. DNA purity can be seen with the A260 / A280 ratio value between 1.8 - 2.0 nm. If <1.8 means contaminated with protein or components of protein derivate contamination that affect DNA molecules and if> 2.0 means contaminated with RNA (Protocol kit).

**3. Amplification and Visualization of the MatK Gen Amplicon**

Stages of PCR were carried out with TopTag Master Mix 12.5 µl, matK_rev2 µl primer and matK_f 2 µl primer, ddH2O 6.5 µl, and DNA template 2 µl and then included in Rotor-Gene Q. Amplicon visualization using automatic electrophoresis Qiaxcel (Qiagen).

**4. Sekuensing**

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Cycle Sequencing is done using BigDye Terminator v3.1 Cycle Sequencing Kit. The sequencing reaction is processed using the ABI 3500 Genetic Analyzer according to the protocol. Sequencing was conducted at First BASE Laboratory, Singapore.

### D. Data Analysis

The sequencing output from Singapore's FIRST BASE in the form of "file.seq" was analyzed using Geneious 10 Bioinformatics software. The reading results with Geneious 10 are sequential chromatograms, basic sequences in nutmeg genes or called sequences and sequence characteristics. The nutmeg gene sequences obtained were used for alignment analysis using the online Barcode Of Life Database (BOLD) Systems (http://www.boldsystems.org). Reconstruction of phylogeny trees using MEGA 7.0 software. The phylogeny tree model used was determined through analysis of a suitable model in MEGA 7.0 software. The phylogeny tree construction uses the Neighbor Joining method with a 1000x boostrap.

## 3. RESULTS AND DISCUSSION

### 3.1 Total DNA Extraction and Purification

DNA extraction and purification using nutmeg leaf tissue (Figure 4.1 a). Nutmeg leaf tissue is prepared using liquid nitrogen (Figure 4.1 b). The powdered samples were extracted using Genomic DNA Mini Kit Plant (Geneaid) based on the kit protocol. Modification is done by soaking Rnase for 10 minutes. Extracted DNA is in the form of clear white liquid (Figure 4.1c). The volume of DNA extracted and purified is 100 µl. Furthermore, as a DNA template for amplification of DNA chloroplast matK genes.

DNA concentration and purity was obtained using a UV / VIS Spectrometer Lambda 35 Perkin Elmer (Table 4.1). DNA concentration of PW1 is 20.3 ($A_{260}/A_{280}$) while purity is 1.6. Compared with PP1 samples, the PP1 DNA concentration was 13.45 ($A_{260}/A_{280}$) while the purity was 2.2 (Table 4.1).

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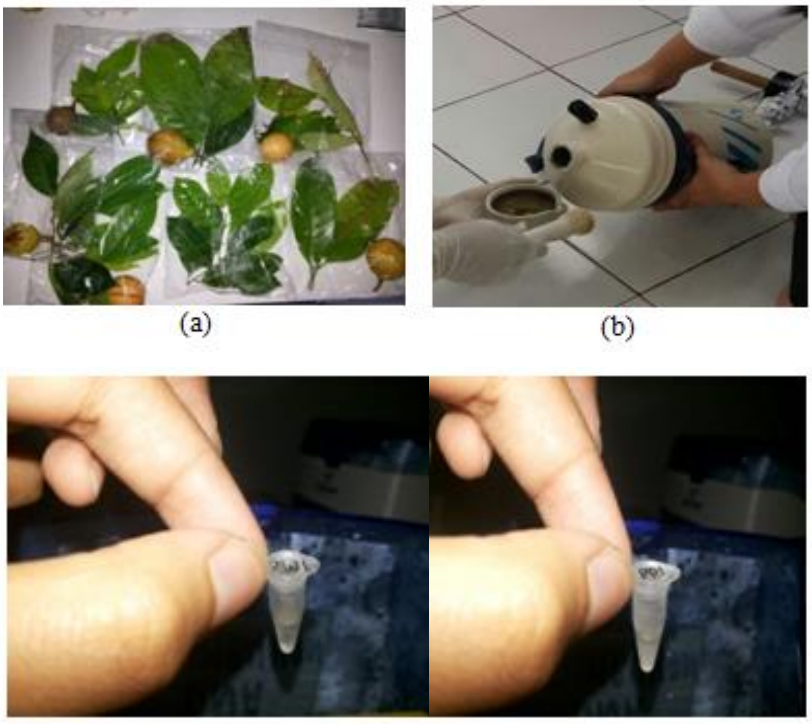


Figure 3. (a) Nutmeg Leaf Sample Tissue. (b) Preparation Using Liquid Nitrogen. (c) Templete DNA (Pala DNA)

Table 4.1 Concentrations and purity of dsDNA from leaf tissue samples

| No | Sample | Sample weight (mg) | Incubation time with Rnase | concentration of dsDNA | Purity | Amount of DNA |
|---|---|---|---|---|---|---|
| 1. | Nutmeg Wori 1 (PW1) | 49,6 mg | 60˚C for 10 minutes | 20,3 | 1,6 | 100 µl |
| 2. | Nutmeg Patokaan 1 (PP1) | 49,2 mg | 60˚C for 10 minutes | 13,45 | 2,2 | 100 µl |

1. Gene Amplification and Visualization of Gen matK Amplification

The PCR reaction was carried out in a total volume of 50 µl. The composition of the PCR reaction is: 25 µl My Taq HS Red Mix, 1 µl primer forward K5' ACCCAGAAATGGATCTCTTCCTGG TTC 3', 1 µl reverse primer K5' CGTACAGTACTT TTGTGTTACGAG 3', 2 µl DNA templates (samples) and 21 µl dlO 21 d2O dd Modified PCR conditions are denaturation at 94 ˚C for 60 seconds, annealing at 50 ˚C for 30 seconds, extension at 72 ˚C for 30 seconds, final extension 72 ˚C for 60 seconds.

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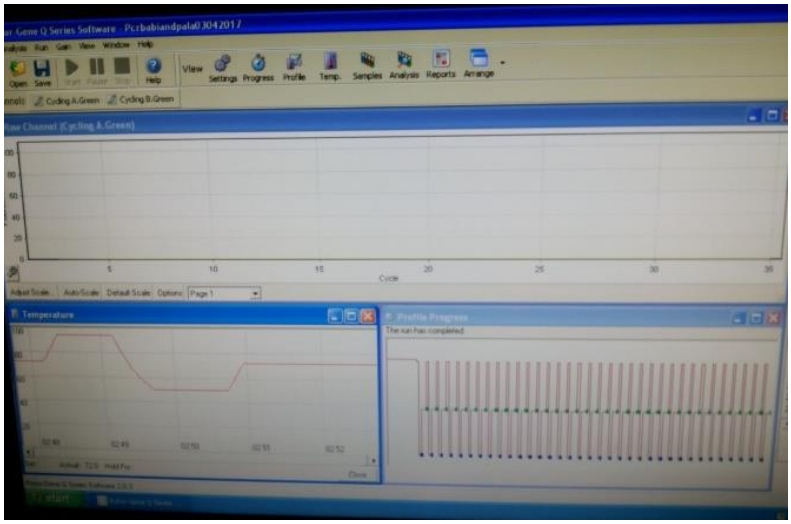

Figure 4. The process of matK gene amplification

After PCR then proceed with electrophoresis using KIT DNA Screening Qiagen (Qiaxel) and work procedures are adjusted with the KIT protocol. Amplification of matK gene for two nutmeg and patokaan nutmeg samples from North Minahasa showed good amplicon results, evidenced by the appearance of bands on the results of PCR visualization using automatic electrophoresis consistently with the absence of smears (bias).

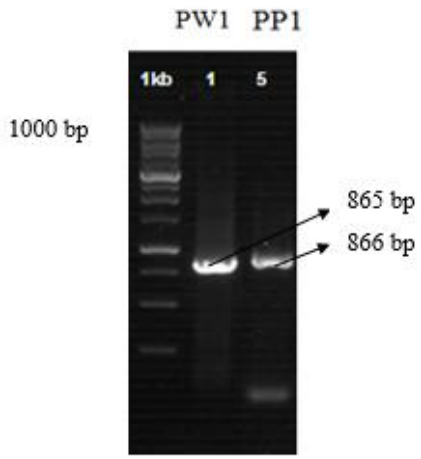


Figure 5. Electrogram amplicon matK genes of PW1 and PP2

### 3.2 Sequencing

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Data sequencing results in the form of seq files. from First Base Singapore were analyzed using the Geneious 10.1.3 program (Drummond et al., 2012), to get the sequence of the North Minahasa nutmeg genes in the FASTA format and then analyzed with the Barcode Of Life Database (BOLD) Systems program. The sample sequences of nutmeg and patokaan nutmeg were 865 bp and 866 bp respectively.

(a)

(b)

Figure 5 (a) Chloroplast DNA Sequences of Wori Nutmeg matK Genes, (b) Chloroplast DNA Sequences of Patokaan Nutmeg matK genes

DNA is extracted from small pieces of leaves of one nutmeg plant using Genomic DNA mini plant (Geneaid) with a modified protocol. Samples were prepared using liquid nitrogen. Chloroplast DNA was extracted using Geneaid Genomic DNA Mini KIT (Plant) by following the KIT protocol. DNA concentration according to the KIT protocol is more than 10 µg and purity is in the range of 1.8-2.0. The results of this study showed that Nutmeg Wori samples incubated with Rnase for 10 minutes produced a concentration of 20.3 µg and purity of 1.6. Whereas Patokaan Nutmeg samples incubated with Rnase for 10 minutes produced a concentration of 13.45 µg and purity of 2.2.

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Total DNA obtained from DNA extraction was further amplified by PCR using TopTag Master Mix and matK primers. Amplification of matK genes Wori Nutmeg and Patokaan Nutmeg with denaturation conditions of 94 ˚C for 45 seconds, anneling 50 ˚C for 55 seconds, extension 72 ˚C for 1 minute, and final extension 72 ˚C for 45 seconds produced unfavorable amplicons in the process of visualization of matK genes with automatic electrophoresis. Modification of PCR components using MyTaq HS Red Mix Bioline with modification of PCR conditions namely denaturation of 94 ˚C for 60 seconds, anneling 50 ˚C for 30 seconds, extension 72 ˚C for 30 seconds, and final extension 72 ˚C for 60 seconds produces amplicon that both through the visualization process with automatic electrophoresis.

Good amplicon is characterized by the formation of a thick black band in visualization using automatic electroforesis, while the less good one is characterized by the absence of a solid black band or a less clear band. The amplification of matK gene for Wori Nutmeg and Patokaan Nutmeg samples from North Minahasa showed good amplicon results evidenced by the appearance of bands on the results of PCR visualization using automatic electrophoresis. Amplicon results are continued in the sequencing process.

## 4. CONCLUSION

The genes of Wori nutmeg and North Minahasa Patokaan nutmeg were amplified with a length of 865 bp and 866 bp. The sequences of Wori nutmeg and Patokaan nutmeg have 99% similarity with *Myristica maingayi* and *Myristica glubosa*, while the reconstruction of phylogeny tree shows that North Minahasa nutmeg has the closest similarity to *Myristica fatua*.

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